THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 45, No. 2

APPLICATION OF THE RYTER-KELLENBERGER FIXATION METHOD TO ELECTRON MICROSCOPIC STUDY OF BACTERIA ON THE SKIN SURFACE*

LEOPOLDO F. MONTES, M.D., DONALD W. OWENS, M.D. AND JOHN M. KNOX, M.D.

Electron microscopic studies of human skin have provided knowledge of the epidermal ultrastructure (1–5). The fine structure of microorganisms is also becoming better understood with the increased use of the electron microscope by microbiologists (6, 7). The widespread use of Ryter and Kellenberger's (R-K) (8) method for the fixation of bacteria has been largely responsible for this progress in microbiology.

Despite these advances very few publications are concerned with the electron microscopic appearance of bacteria growing in the skin (9–11). The present report relates the results obtained following the application in our laboratory of the R-K technic for the observation of bacteria present on the skin surface. The dermatological use of such a method arose from the need to overcome difficulties in obtaining a satisfactory bacterial fixation from human skin when using the conventional methods for mammalian tissues.

MATERIAL AND METHODS

Skin specimens from the crural region of five erythrasma patients, from the interdigital spaces between the toes of one patient with intertrigo, and from the anterior right forearm of two normal individuals were used in this study. These specimens were known to contain bacteria because positive bacterial cultures were obtained either by swabbing or scraping the same areas that were removed for biopsy.

Following injection of 2 cc of 2 per cent xylocaine into the subcutis, to avoid distortion of the epidermis, 2 mm punch biopsy specimens were removed. The specimens included only the epidermis and the upper dermis. Immediately after removal, the specimens were immersed in the Ryter-Kellenberger (8) fixative. Shortly thereafter the specimens being fixed were placed under a stereomicroscope and cut into smaller pieces, about 0.25 cubic mm. Most of the remaining dermis was also removed at this time with a razor blade.

The pre-fixation step as suggested in the original method for bacterial cultures was eliminated. However, the main steps of the technic were carefully followed. These are: 1) fixation in a 1 per cent O_sO_4 solution in veronal buffer (pH 6) for 16 hours, at room temperature; 2) addition of Ca and tryptone to the fixative; 3) washing in the veronal buffer following the fixation period and subsequent treatment with 0.5 per cent uranyl acetate; 4) dehydration in acetone. Because we were dealing with cubes of skin, the pre-embedding in soft agar was not necessary. Final embedding was done using a mixture of Epon and Araldite.

A Porter-Blum ultramicrotome equipped with a diamond knife was used to obtain ultrathin sections by cutting the skin perpendicularly to the surface. An RCA EMU3F electron microscope with an accelerating voltage of 50 kv was used to observe and photograph the material.

In every case one biopsy specimen, or part of it, was fixed in formalin and embedded in paraffin. Four-micron sections from these blocks were stained with hematoxylin and eosin and with the MacCallum-Goodpasture stain for bacteria in tissue (12). These sections were used for observations at the light microscope level.

OBSERVATIONS

Bacteria present on the skin surface were easily localized when working at low magnifications, mainly because of their characteristic size and shape and also because of the thickness and electron density of their cell walls (Fig. 1). As the magnification was increased the different features of the fine bacterial structure became apparent (Fig. 1). The *cell wall* was usually electron-dense, its thickness ranging between 200–500 Å. Different degrees of electron density were seen so there was not a uniform pattern. The double-layered type of wall described in gram negative organisms (13, 14, 15) was often seen. The *plasma membrane* was well apparent in some bacteria and difficult to observe in others. Membranous structures connected

Received for publication August 1, 1964.
* From the Department of Dermatology, Baylor University College of Medicine, Houston, Texas.
The study reported herein was supported in part by research grants from the Eli Lilly Co. and the Upjohn Co.
David Taplin, Instructor of Dermatology at the University of Miami School of Medicine, performed the cultures for some of the patients used in this study.

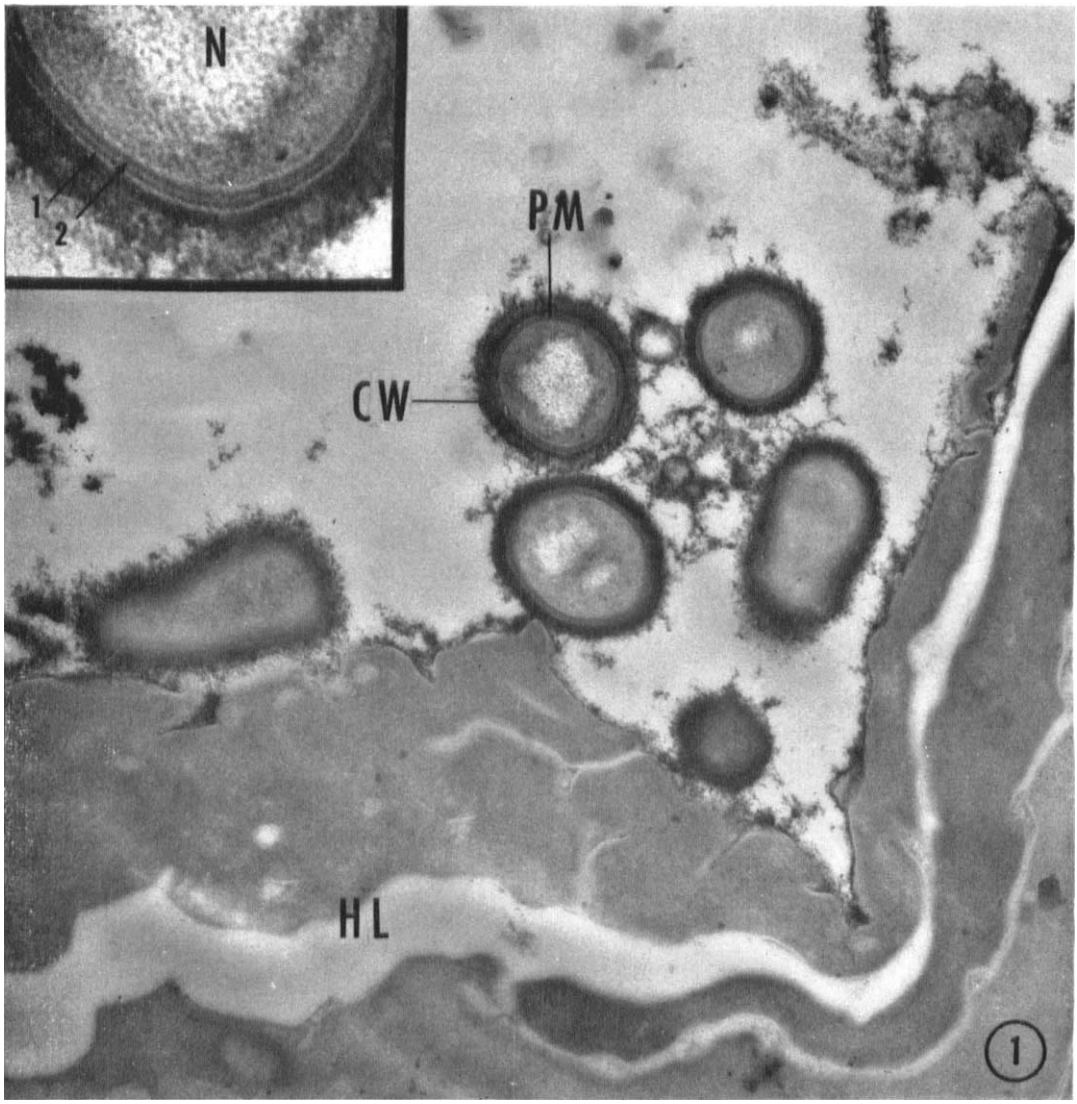

FIG. 1. The skin surface of a patch of erythrasma in the groin, showing several bacteria on the skin. Some loose scales of the superficial horny layer (HL) are seen. The cell wall (CW), capsule (e), plasma membrane (PM) and nucleoplasm (N) are well preserved (× 29,700). Inset shows at higher magnification the double-layered cell wall (1–2) of one of the same bacteria (× 135,000).

to the plasma membrane were observed in the peripheral cytoplasm of many bacteria (Fig. 2, 3). These organelles were thought to represent the so-called mesosomes (16) or chondrioides (8), the equivalents of mitochondria in bacteria (17, 18). The *cytoplasm* or most of the bacteria was also adequately fixed. As observed in cultures (8) there were dark granules, 100–150 Å in diameter, probably representing ribonucleoprotein particles. The lighter zones surrounding them described by others (8) also were seen. Cytoplasmic inclusions, such as those believed to be lipids (19, 20) and represented by round areas of low electron density, were only occasionally seen. The *nucleoplasm* was observed in many favorable sections (Figs.

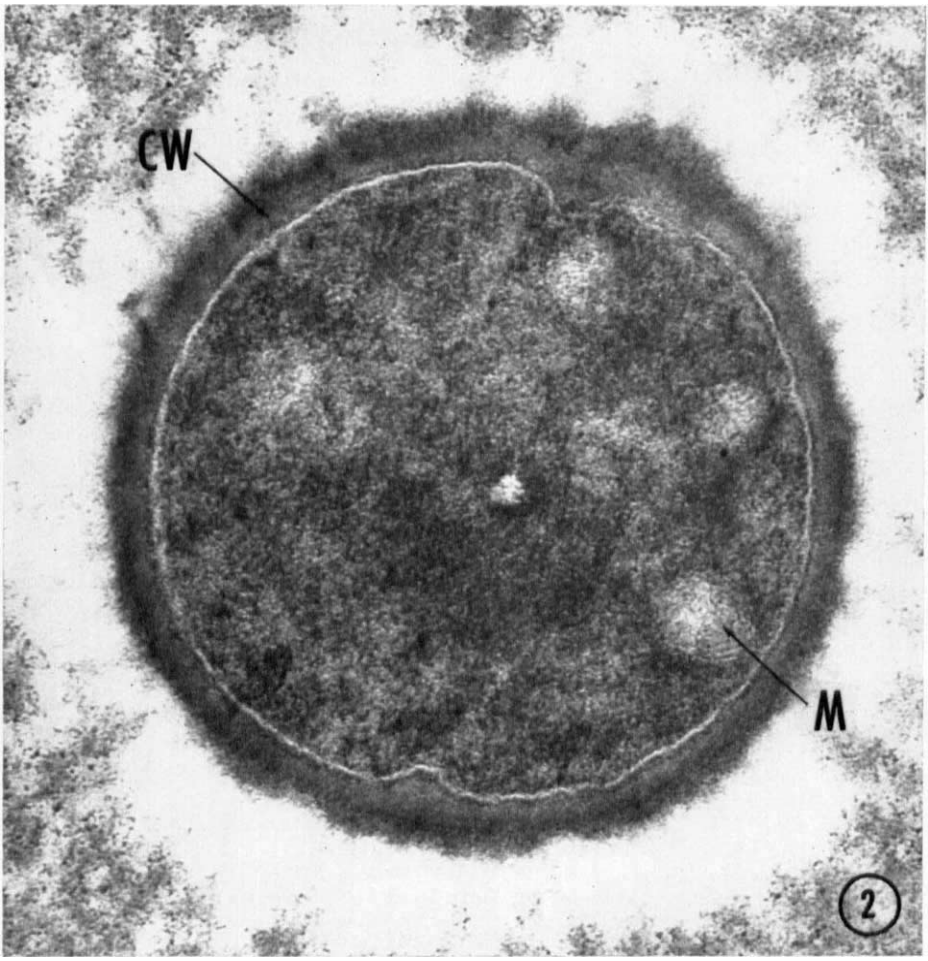

FIG. 2. This bacterium was observed on the skin surface in a specimen from a patient with intertrigo. The cell wall (CW) is well preserved. The plasma membrane is not well defined. A few round areas of low density barely visible in the periphery of the cytoplasm seem to represent mesosomes (M) (× 149,800).

1, 4). A fine and homogeneous structure of the type described by Ryter and Kellenberger was detected (Fig. 4).

Different features of cell division could be studied in bacteria growing on the skin surface. In Fig. 4 the nuclear division preceded an inward growth of the cell wall across the cytoplasm.

The *epidermis* itself showed signs of overfixation. This effect was less pronounced in the horny layer with cells, normally devoid of numerous cytoplasmic organelles. In the malpighian layer many organelles had a washed-out appearance although they were still recognizable. Comparative results of bacterial fixation with R-K technic and other methods will be reported in detail elsewhere (21).

DISCUSSION

It is well known (6–8) that the R-K method applied to the fixation of bacteria growing in cultures is much more effective than other electron microscopic fixation technics. Comparable results are obtained when skin specimens containing bacteria are fixed in the same way. Although the epidermal cells showed

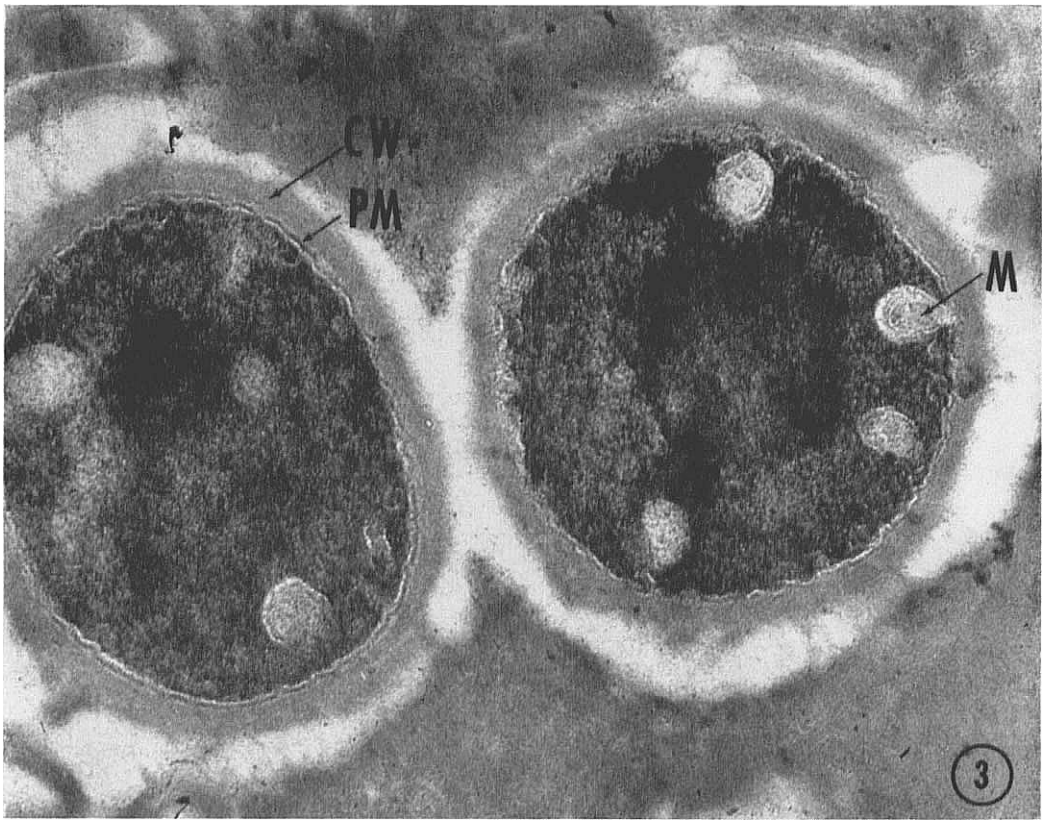

FIG. 3. These bacteria were observed between loose superficial cells of the horny layer from the same patient as illustrated in Fig. 2. The cell wall (CW) has less density than the cytoplasm. Several rounded areas (M) lower in density than the rest of the cytoplasm seem composed of infoldings of the plasma membrane (PM) (× 77,760).

signs of overfixation, most of the bacteria present on the skin surface were beautifully preserved.

Our knowledge of bacterial infections of the skin at the submicroscopic level is still very poor. It seems likely that the fixation method described by Ryter and Kellenberger will enable a better understanding of problems relating to cutaneous infections.

An attempt is being made in our laboratory to trace the different stages of horny layer penetration by diphtheroids in erythrasma. We have been able to confirm already the early visualization by Sarkany, Taplin and Blank (9), of bacteria between the layers of the stratum corneum and within the keratinized cells.

No attempt was made to identify the organisms seen on the skin, on the basis of their electron microscopic appearance. In the erythrasma patients, however, because of the clinical diagnosis confirmed by cultures, many of the bacteria were considered to be *C. minutissimum* (22, 23).

SUMMARY

Skin specimens containing bacteria were fixed using the Ryter-Kellenberger method. Electron microscopic observation of this material revealed that bacteria present on the skin surface were well preserved. The different features of the fine bacterial structure could be clearly observed.

REFERENCES

1. Odland, G. F.: The fine structure of the interrelationship of cells in the human epidermis. J. Biophys. Biochem. Cytol., *4:* 529, 1958.
2. Charles, A. and Ingram, J. T.: Electron microscope observations of the melanocyte of the

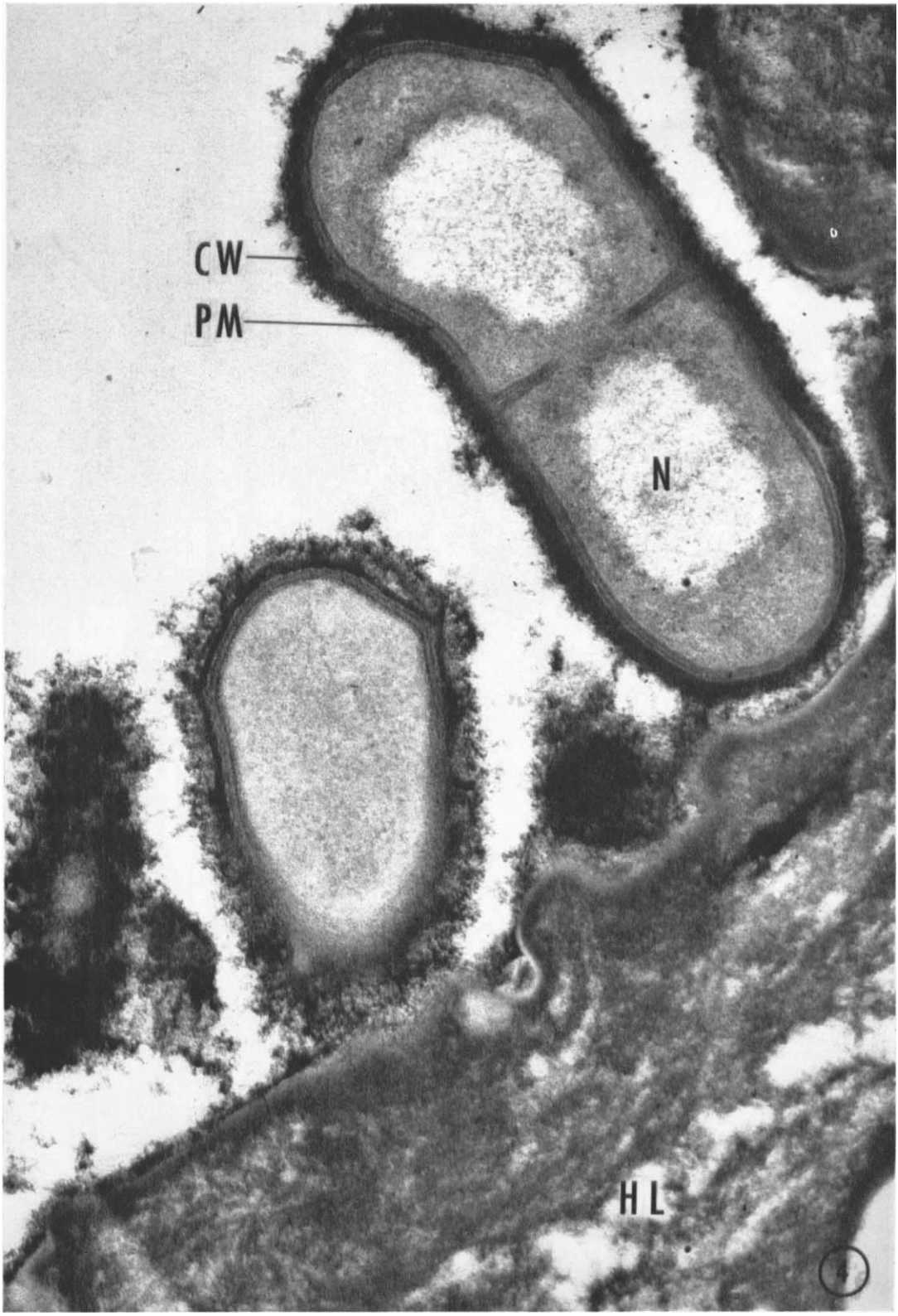

FIG. 4. The skin surface in a case of erythrasma seen at higher magnification than in Fig. 1. Several features of cell division are shown. A new septum is forming across the cytoplasm. The nucleoplasm (N) is already divided in two portions. Ribosomes-like particles are also seen (R) (× 62,100).

human epidermis. J. Biophys. Biochem. Cytol., *6:* 41, 1959.
3. Hibbs, R. G. and Clark, Jr., W. H.: Electron microscope studies of the human epidermis, the cell boundaries and topography of the stratum malpighii. J. Biophys. Biochem. Cytol., *6:* 71, 1959.
4. Brody, I.: The ultrastructure of the tonofibrils in the keratinization process of normal human epidermis. J. Ultrastruct. Res., *4:* 264, 1960.
5. Zelickson, A. S. and Hartmann, F. J.: An electron microscopic study of human epidermis. J. Invest. Derm., *36:* 65, 1961.
6. Brieger, E. M.: Structure and Ultrastructure of Microorganisms. New York and London, Academic Press, 1963.
7. Gunsalus, I. C. and Stanier, R. Y.: The Bacteria—A Treatise on Structure and Function. Vol. I, Structure. New York and London, Academic Press, 1960.
8. Ryter, A. and Kellenberger, E.: Etude Au Microscope Electronique de Plasmas Contenant de l'Acide Desoxyribonucleique. Z. Naturforsch., *13B:* 597, 1958.
9. Sarkany, I., Taplin, D. and Blank, H.: The etiology and treatment of erythrasma. J. Invest. Derm., *37:* 283, 1961.
10. Convit, J. and Imaeda, T.: Electron microscope study of mycobacterium leprae and its environment in a vesicular leprous lesion. J. Bact., *83:* 43, 1962.
11. Chapman, G. B.: Fine structure of the human wart. Amer. J. Path., *42:* 619, 1963.
12. Mallory, F. B.: Pathological Technique, p. 274. Philadelphia, W. B. Saunders Co., 1942.
13. Kellenberger, E. and Ryter, A.: Cell wall and cytoplasmic membrane of escherichia coli. J. Biophys. Biochem. Cytol., *4:* 323, 1958.
14. Claus, G. W. and Roth, L. E.: Fine structure of the gram-negative bacterium acetobacter suboxydans. J. Cell Biol., *20:* 217, 1964.
15. Bladen, M. A. and Waters, J. F.: Electron microscopic study of some strains of bacteroides. J. Bact., *80:* 1339, 1963.
16. Fitz-James, P. C.: Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. J. Biophys. Biochem. Cytol., *8:* 507, 1960.
17. Van Iterson, W. and Leene, W.: A cytochemical localization of reductive sites in a gram positive bacterium. J. Cell Biol., *20:* 361, 1964.
18. Van Iterson, W. and Leene, W.: A cytochemical localization of reductive sites in a gram negative bacterium. J. Cell Biol., *20:* 377, 1964.
19. Wyss, O., Neumann, M. and Socolofsky, M. D.: Development and germination of the azotobacter cyst. J. Biophys. Biochem. Cytol., *10:* 555, 1961.
20. Gale, G. and McLain, H.: Effect of ethambutol on cytology of mycobacterium smegmatis. J. Bact., *86:* 749, 756, 1963.
21. Montes, L. F.: Unpublished Study.
22. Munro-Ashman, D., Wells, R. S. and Clayton, Y. M.: Erythrasma in adolescence. Brit. J. Derm., *75:* 401, 1963.
23. Sarkany, I., Taplin, D. and Blank, H.: Organism causing erythrasma. Lancet, *2:* 304, 1962.